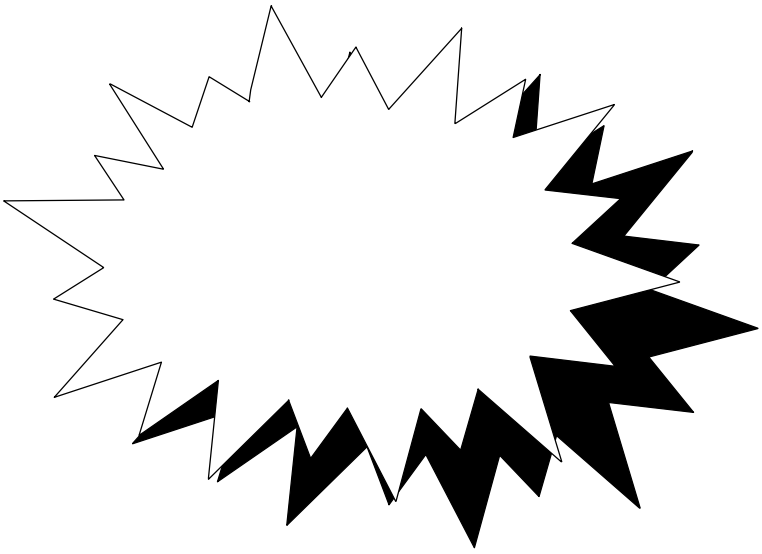

Chemical Analysis and Testing Task

Laboratory Analytical Procedure

LAP-013

Procedure Title: HPLC Analysis of Liquid Fractions of Process Samples for Monomeric Sugars and Cellobiose

Author: Raymond Ruiz and Tina Ehrman

Date: 6/28/96

ISSUE DATE: 8/26/96 **SUPERSEDES:** 11/01/94

DISCLAIMER

These Standard Biomass Analytical Methods (“Methods”) are provided by the National Renewable Energy Laboratory (“NREL”), which is operated by the Midwest Research Institute (“MRI”) for the Department Of Energy.

Access to and use of these Methods shall impose the following obligations on the user. The user is granted the right, without any fee or cost, to use, copy, modify, alter, enhance and distribute these Methods for any purpose whatsoever, except commercial sales, provided that this entire notice appears in all copies of the Methods. Further, the user agrees to credit NREL/MRI in any publications that result from the use of these Methods. The names NREL/MRI, however, may not be used in any advertising or publicity to endorse or promote any products or commercial entity unless specific written permission is obtained from NREL/MRI. The user also understands that NREL/MRI is not obligated to provide the user with any support, consulting, training or assistance of any kind with regard to the use of these Methods or to provide the user with any updates, revisions or new versions.

THESE METHODS ARE PROVIDED BY NREL/MRI "AS IS" AND ANY EXPRESS OR IMPLIED WARRANTIES, INCLUDING BUT NOT LIMITED TO, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE ARE DISCLAIMED. IN NO EVENT SHALL NREL/MRI BE LIABLE FOR ANY SPECIAL, INDIRECT OR CONSEQUENTIAL DAMAGES OR ANY DAMAGES WHATSOEVER, INCLUDING BUT NOT LIMITED TO CLAIMS ASSOCIATED WITH THE LOSS OF DATA OR PROFITS, WHICH MAY RESULT FROM AN ACTION IN CONTRACT, NEGLIGENCE OR OTHER TORTIOUS CLAIM THAT ARISES OUT OF OR IN CONNECTION WITH THE ACCESS, USE OR PERFORMANCE OF THESE METHODS.

HPLC Analysis of Liquid Fractions of Process Samples for Monomeric Sugars and Cellobiose

Laboratory Analytical Procedure #013

1. Introduction

1.1 Carbohydrates make up a major portion of biomass samples. These carbohydrates are polysaccharides constructed primarily of glucose, xylose, arabinose, galactose, and mannose monomeric subunits. During the processing of the biomass, such as dilute acid pretreatment, a portion of these polysaccharides are hydrolyzed and soluble sugars released into the liquid stream. Fermentation samples, whether they are time point samples or end point residues, will also contain soluble sugars. The soluble sugars in the liquid fraction of process samples can be quantified by HPLC with refractive index detection.

2. Scope

2.1 This procedure is used to determine the soluble monosaccharide content of the liquid fractions of biomass to ethanol process streams, including pretreatment liquors, liquid fermentation time point samples, and the liquid fraction of fermentation residues. The soluble sugar content indicates the amount of fermentable sugars available for conversion to ethanol at specific process steps.

2.2 All analyses shall be performed according to the guidelines established in the Ethanol Project Quality Assurance Plan (QAP).

3. References

3.1 Ehrman, C.I., and M.E. Himmel. 1994. "Simultaneous Saccharification and Fermentation of Pretreated Biomass: Improving Mass Balance Closure." *Biotechnology Techniques*, 8(2):99-104.

3.2 Moore, W., and D. Johnson. 1967. Procedures for the Chemical Analysis of Wood and Wood Products. Madison, WI: U.S. Forest Products Laboratory, U.S. Department of Agriculture.

4. Significance and Use

4.1 The concentrations of monomeric sugars and cellobiose are used in conjunction with other assays to determine the total composition of process stream samples.

5. Apparatus

5.1 Analytical balance, accurate to 0.1 mg.

5.2 pH meter, readable to 0.01 pH unit.

5.3 HPLC system equipped with refractive index detector.

5.4 Biorad Aminex HPX-87C and/or HPX-87P columns (or analytical HPLC columns shown to give equivalent separations as the Biorad columns). equipped with the appropriate guard columns.

> Note: Deashing guard column cartridges from BioRad, of the ionic form H^+/CO_3^-, are recommended when using an HPX-87P column. These cartridges have been found to be effective in eliminating baseline ramping.

6. Reagents and Materials

6.1 High purity sugars for standards - cellobiose, glucose, xylose, arabinose, galactose, and mannose.

6.2 Second set of the high purity sugars listed above, from a different source (manufacturer or lot) for preparation of calibration verification standards (CVS).

6.3 Calcium carbonate, ACS reagent grade.

6.4 Water, HPLC grade or better, 0.2 μm filtered.

6.5 pH paper (range 2-9).

6.6 Disposable syringes, 3 mL, fitted with 0.2 μm syringe filters.

6.7 Autosampler vials with crimp top seals to fit.

6.8 Volumetric pipets, class A, of appropriate sizes.

6.9 Volumetric flasks, class A, of appropriate sizes.

6.10 Adjustable pipettors, covering ranges of 10 to 1000 µL.

7. ES&H Considerations and Hazards

7.1 Follow all applicable NREL Laboratory Specific Hygiene Plan guidelines.

8. Calibration and Standardization

8.1 This analysis uses a multipoint calibration as described in the procedure.

9. Procedure

9.1 Thoroughly mix the sample and then measure and record the pH of a small aliquot to the nearest 0.01 pH unit. It will be necessary to adjust the pH of the sample if this reading falls outside of the operating pH range (5-9) of the HPLC column to be used.

9.2 Dilute the samples as needed, so the concentration of each sugar falls within the validated range of the analytical method. Prepare each dilution in duplicate.

> Note: It may be useful to determine initial glucose concentrations of the samples using an alternative technique, such as YSI glucose analyzer, in order to predict whether or not the sugars in the sample will fall within the linear range of the analysis. In samples such as pretreatment liquors, xylose typically is present at high concentrations, often three to five times the level of glucose. In other samples, such as the liquid fractions of fermentation samples, the levels of all the soluble sugars can be relatively low, and the samples therefore will not require dilution.

9.3 Prepare method verification standards (MVS) by selecting representative samples to be used to determine spike recoveries. Spike an accurately measured volume of each selected sample with a known amount of the components of interest, such that the final concentrations still fall within the linear range of the analysis. Process these spiked samples along with the rest of the samples.

9.4 If the pH of the sample is less than 5, neutralize duplicate 10 mL aliquots with calcium carbonate to a pH between 5 and 6. Avoid neutralizing to a pH greater than 6 by monitoring with a pH strip. Add the calcium carbonate slowly after about pH 4 and swirl frequently.

> Note: Samples of pH greater than 9, such as alkaline pretreatment liquors, should not be analyzed using the HPX-87C or HPX-87P columns unless the sample pH can be brought into the operating range of the column, pH 5 to 9.

9.5 Pass the appropriately diluted and/or neutralized samples through 0.2 µm syringe filters into autosampler vials in preparation for HPLC analysis. Seal and label the vials. Reserve a portion of the undiluted sample in case repeat analyses are required.

Store the reserved samples in the refrigerator.

9.6 Prepare a series of sugar calibration standards in HPLC grade water at concentrations appropriate for creating a calibration curve for each sugar of interest. A suggested scheme for the HPX-87C column is to prepare a set of multi-component standards containing glucose, xylose, and arabinose in the range of 0.2 - 12.0 mg/mL. For the HPX-87P column, cellobiose, galactose, and mannose should be included as additional components in the standards. Extending the range of the calibration curves beyond 12.0 mg/mL will require validation.

9.7 Prepare an independent calibration verification standard (CVS) for each set of calibration standards, using sugars obtained from a source other than that used in preparing the calibration standards. The CVS must contain precisely known amounts of each sugar contained in the calibration standards, at a concentration that falls in the middle of the validated range of the calibration curve. The CVS is to be analyzed after each calibration curve and at regular intervals in the HPLC sequence, bracketing groups of samples. The CVS is used to verify the quality of the calibration curve(s) throughout the HPLC run.

9.8 Analyze the calibration standards, the calibration verification standards, the samples, and the method verification (spiked) samples by HPLC using a Biorad Aminex HPX-87C or HPX-87P column for glucose, xylose, and arabinose. If cellobiose, mannose, and galactose are also to be determined, only the Biorad Aminex HPX-87P column should be used. For many analyses, it is useful to run the same samples on both columns and compare the results. The following instrumental conditions are used for both the HPX-87C and the HPX-87P columns:

Sample volume: 50 μL.
Eluant: 0.2 μm filtered and degassed HPLC grade water.
Flow rate: 0.6 mL/min.
Column temperature: 85^{o}C.
Detector: refractive index.
Run time: 20 minutes data collection plus a 15 minute post-run.

10. Calculations

10.1 Create a calibration curve for each sugar to be quantified using linear regression. From these curves, determine the concentration in mg/mL of the sugars present in each sample analyzed by HPLC, corrected for dilution.

10.2 Calculate and record the percent recoveries, %CVS, for each sugar contained in the calibration verification standards analyzed by HPLC.

$$\%\ CVS\ recovered = \frac{conc.\,detected\ by\ HPLC, mg/ml}{known\ conc.\,of\ \ CV\ before\ HPLC\ analysis, mg/ml} \ x\ 100$$

10.3 Calculate and record the percent spike recoveries (% recovery MVS) for each sugar used to prepare the method verification standards analyzed by HPLC.

10.3.1 Correct the initial sample concentration for the dilution resulting from the addition of a known volume of spike solution.

$$C_{corrected} = \frac{V_{sample}}{V_{final}} \ x\ C_{sample}$$

Where: V_{sample} = volume of sample prior to spiking, in mL.
V_{final} = final volume of solution (spike plus sample), in mL.
C_{sample} = initial concentration of sample prior to spiking in mg/mL, as determined by HPLC.
$C_{corrected}$ = concentration of sample after being corrected for dilution, in mg/mL.

10.3.2 Calculate the percent recovery of the spike.

$$\%\ Recovery\ MVS = \frac{C_{actual} - C_{corrected}}{C_{spike}} \ x\ 100$$

Where: C_{actual} = actual concentration of spiked sample, as determined by HPLC, in mg/mL.
$C_{corrected}$ = concentration of sample after correcting for dilution, in mg/mL, as calculated above.
C_{spike} = known concentration of spike solution added to sample prior to analysis, in mg/mL.

11. Precision and Bias

11.1 In the determine the cellobiose and monomeric sugar contents of process samples, the neutralized samples are routinely analyzed using the HPX-87P column. When these samples are known not to contain galactose and mannose, the HPX-87C column may be used instead. Based on a root mean square evaluation of duplicate data, there is a 95% certainty that the "true value" will be within the range of the average plus or minus:

- glucose 3.37% (HPX-87C) and 3.12% (HPX-87P),
- xylose 1.92% (HPX-87C) and 5.02% (HPX-87P).

Analytes at or near the detection limit could have significantly higher precision errors.

11.2 Samples containing significant amounts of protein, oligosaccharides, or other compounds which elute early on the HPX-87C and HPX-87P columns will exhibit baseline disturbances which may interfere with the quantification of the analytes.

12. Quality Control

12.1 *Reported significant figures:* Report all results in mg/mL with two decimal places. The standard deviation and relative percent difference are also to be reported.

12.2 *Replicates:* All samples are to be run in duplicate. For replicate analyses of the same sample, report the average, standard deviation, and relative percent difference (RPD).

12.3 *Relative percent difference criteria:* The maximum RPD for duplicate samples is as follows: glucose, 5.8%, and xylose, 8.1%. If the stated RPD is exceeded, the sample should be rerun. However, analytes at or near the detection limit could have significantly higher RPDs.

12.4 *Blank:* The only requirement is an instrumental blank, consisting of the HPLC grade water analyzed by HPLC in the same manner as the samples.

12.5 *Method verification standard:* This method will utilize a matrix spike as the method verification standard, as indicated in the procedure.

12.6 *Calibration verification standard:* Calibration verification standards shall be independently prepared and analyzed as described in the procedure.

12.7 *Definition of a batch:* Any number of samples which are analyzed together and recorded together. Samples within a batch must be of the same matrix. The maximum size of a batch will be limited by the equipment constraints. A batch cannot be larger than what is practical with the equipment.

12.8 *Sample size:* 30 mL minimum.

12.9 *Sample storage:* Samples should be refrigerated.

12.10 *Standard storage:* Standards should stored frozen. Upon thawing, the standards should be vortexed p and then shaken after thawing and again prior to use.

12.11 *Standard preparation:* Standards are prepared according to section 8.6 of this procedure.

12.12 *Control charts:* All spike recoveries and calibration verification standards are control charted.